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Isolation and Characterization of Two Virulent Phages to Combat *Staphylococcus aureus* and *Enterococcus faecalis* causing Dental Caries

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Abstract

This study aimed to isolate and characterize bacteriophages, as a biocontrol agent, against certain antibiotic-resistant bacteria causing dental caries. Here, two dental caries-causing bacteria *S. aureus* and *E. faecalis* were isolated and characterized biochemically using the automated VITEK® 2 system. Antibiotic sensitivity pattern of the isolated dental caries bacteria was assessed against selection of antibiotics. The two isolates showed resistance against most of the tested antibiotics. To overcome this problem, two lytic phages vB_SauM-EG-AE3 and vB_EfaP-EF01 were isolated, identified, and applied to control the growth of *S. aureus* and *E. faecalis,* respectively. Phages were identified morphologically using TEM and showed that vB_SauM-EG-AE3 phage is related to *Myoviridae* and vB_EfaP-EF01 phage belongs to *Podoviridae*. The two phages exhibited high lytic activity, high stability, and a narrow host range. The one-step growth curve of phages showed burst sizes of 78.87 and 113.55 PFU/cell with latent periods of 25 and 30 minutes for *S. aureus* phage and *E. faecalis* phage respectively. In addition, the two phages showed different structural protein profiles and exhibited different patterns using different restriction enzymes. The genome sizes were estimated to be 13.30 Kb and 15.60 Kb for phages vB_SauM-EGAE3, vB_EfaP-EGAE1, respectively. Complete inhibition of bacterial growth was achieved using phages with MOIs of 10^3, 10^2 and 10 after 1, 3, 5, and 24 h of incubation at 37°C. Hence, this study indicates that the isolated bacteriophages are promising biocontrol agents that could challenge antibiotic-resistant dental caries bacteria to announce new successful alternatives to antibiotics.

Keywords: Dental caries, *Enterococcus faecalis*, *Staphylococcus aureus*, multidrug-resistance, bacteriophage, biocontrol

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INTRODUCTION

Enterococcus is a genus of Gram-positive, non-spore-forming bacteria commonly found in the oral cavity, gastrointestinal tract, and vagina[1]. Though most species of *Enterococcus* in humans and animals are considered commensal[2]. Emergence of Enterococcus as human pathogens due to the presence of virulence factors such as adhesive production and aggregation compounds, or biogenic amine production in fermented foods[3] and they resist different measures of disinfection and create a biofilm[4]. *Enterococcus faecalis* (*E. faecalis*) was not considered to belong to the normal oral flora. *E. faecalis* can act as an opportunistic pathogen that causes bacteremia, meningitis, endocarditis, or urinary tract and wound infection[5,6]. It has several survival mechanisms for living in unfavorable environments, such as grow in a low-oxygen environment at high pH, a broad range of temperatures between 10°C and 60°C and high salinity or a poor nutritional environment[7]. In patients with post-treatment apical periodontitis or refractory apical periodontitis, *E. faecalis* was located mainly in the root canals, suggesting an etiological role in the development of these diseases[4,8]. The excessive use or abuse of antibiotics has triggered a disturbing appearance of virulent antibiotics resistant pathogenic bacteria[9]. As important nosocomial pathogens, the therapeutic challenge of multi-drug resistant enterococci (MDR) strains with substantial resistance to two or more antibiotics, often including, though not limited to, vancomycin, has brought their role to a sharper focus[10].

Staphylococcus aureus (*S. aureus*) is a gram-positive coccoid bacterium that causes human bacteremia, endocarditis, osteoarticular, skin and soft tissue, pleuropulmonary infection, and device-related infections[11]. *S. aureus* was isolated from the oral cavity[12]. Many oral infections are at least partly caused by *S. aureus*, as parotitis, and staphylococcal mucositis[13]. *S. aureus* has a range of virulence factors including adhesives, toxins, coagulase, and a variety of genes for resistance to antimicrobials[14]. Clinical *S. aureus* isolates exhibit recurrent multidrug resistance, with the most important being methicillin and vancomycin resistance[15]. Mature biofilm formation induces additional antibiotic tolerance[16]. Infections with multidrug-resistant *S. aureus* require new therapies with current interest focused on bacteriophages (phages).

Bacteriophages (phages) are viruses that invade bacteria; they interrupt the metabolism of bacteria and cause lysis of bacterial cells (lytic phages). Specifically, each type of phage attacks only certain bacteria as its host; it frequently does not affect total microbial biomass[17]. During-active infection, a strictly virulent phage generates typically more than 100 copies of itself[18]. If the numberof phages is lower than that of bacterial cells, after many generations the population of phages will surpass that of bacterial cells, ultimately the total bacterial cells will lyse[17].

One alternative that has recently revived interest is phage therapy, first proposed in the early 20th century by Felix d'Herelle. Besides, several clinical studies have shown that the use of bacteriophages in both humans and animals is effective and without side effects[19,20]. Lytic phages are considered as potential alternative candidates to conventional antibiotics for a wide range of oral bacterial infections[21].

The first phage therapy was reported concerning the treatment of *S. aureus* skin infections; also, phage therapy was used in some infections associated with burn wounds caused by *S. aureus*. Phages have been reported to be successful in treating various bacterial diseases such as infections of the skin caused by Staphylococcus, neonatal sepsis, infections of the urinary tract, and diabetic foot as a complication of diabetes[22].

Polyvalent bacteriophage K, a well-characterized member of the *Myoviridae* family of viruses, is a candidate for combating *S. aureus* MDR infections[23]. Estrella et al(24) isolated and identified seven novel phages with broad lytic activities for *S. aureus* from untreated sewage. A phage able to parasitize *E. faecalis* has been described[25]. A total of 23 lytic phages, targeting *E. fecalis*, are based on previously published studies. They were isolated from several sources, including wastewater, effluent from farm animals and human feces[26,21,27.]

In vitro antimicrobial effect bacteriophages on human dentin infected with *E. faecalis* ATCC 29212 indicated that applying bacteriophage

lysate to the roots after 6days incubation resulted in a major decrease in the survival of the bacteria[28]. Lee et al[21] indicated that bacteriophage HEf13 has a lytic activity and is a therapeutic potential agent for treating or preventing infectious diseases associated with *E. faecalis*[21]. In this study, bacteriophage isolation and identification were achieved as novel candidates appropriate for bacteriophage therapy against *E. faecalis* and *S. aureus* as alternatives to conventional antibiotics.

MATERIALS AND METHODS

Bacterial strains and growth conditions

This study was performed on two antibiotic-resistant dental caries isolates that were isolated previously from patients with dental plaques in Qalubiya governorate, Egypt. All strains were stored at -80°C in Brain-Heart-Infusion broth complemented with 20% (v/v) glycerol. Freshly overnight grown cultures were prepared by inoculating a single colony into 10 ml of Basal salt medium with yeast extract and incubating for 16 h at 37°C with shaking at 200 rpm.

Morphological and Biochemical identification of the dental caries bacteria

Morphological and Biochemical identification (Table S1, Supplementary data) of the dental caries bacteria were carried out according to Bergey's Manual of Systematic Bacteriology. These isolates were confirmed by VITEK® 2 COMPACT automated instrument for ID/AST testing[29].

Antibiotic sensitivity test

Antibiotics sensitivity testing was performed on Mueller-Hinton agar by the disc diffusion method[30] for the following antibiotics (Oxoid, UK); Nitrofurantoin (F, 10 µg), Amoxicillin (AX, 25 µg), Chloramphenicol (C, 30 µg), Ciprofloxacin (CIP, 5 µg), Gentamicin (GN, 10 µg), Norfloxacin (NOR, 10 µg), Penicillin-G (P, 10 µg), Vancomycin (V, 30 µg), Ceftazidime (CAZ, 30 µg), Aztreonam (ATM, 30 µg), Cefotaxime (CTX, 30 µg) and Amikacin (AK, 30 µg). The results were interpreted conferring to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[31].

Isolation of Bacteriophages

The clinical isolates *S. aureus* EG-AE3 and *E. faecalis* EF01 were used as hosts for the isolation, and propagation of bacteriophages. Saliva samples from 15 healthy individuals and 85 dental caries affected patients and drainage samples from dental clinics were collected from Benha city, Qalubiya governorate, Egypt for bacteriophage screening. The collected samples were centrifuged at 10,000 rpm for 10 min, supernatant fluids were collected, and membrane-filtered using 0.22 µm membrane filters (Millipore, Ireland).

Enrichment of phages and isolation were performed as described previously[32]. Briefly, 5 ml of a 0.22 µm-filtered sample was mixed with 20 ml double-strength Tryptic Soybean Broth (TSB) medium and 100 µl of a mid-log culture of each of *S. aureus* strain EG-AE3 and *E. faecalis* EF01 and incubated for 48 h at 37°C with shaking at 200 rpm. Later, bacteria were harvested by centrifugation at 10,000 rpm for 10 min, supernatant fluids were recovered, and membrane-filtered using 0.22-µm Millipore filters (Millipore, Ireland). Phages were screened by spotting five microliters of the enriched samples onto double-layered plates containing a lawn of the indicator bacteria strain and incubated for 48 h at 37°C[33]. Plates were inspected for the presence of clear lysis zones; the clear zone was cut and propagated in a fresh culture. This lysate was serially diluted, spotted onto double-layered plates, and incubated as described above.

Transmission electron microscopy of bacteriophages

Ten microliters of highly purified phage suspension were fixed onto 300- by 300-mesh copper grids (Electron Microscopy Sciences) supported by carbon-coated Formvar film[34]. After 5 minutes, fixed phages were negatively stained with 2% (w/v) aqueous phosphate tungsten acid, pH 7.2 for 1 min, and air-dried at room temperature for 1 h. Transmission electron microscope (A JEOL JEM-2100) was used for attaining the phage particle images at the Electron Microscope Facility, Al-Azhar University, Egypt.

One-step growth curve

Phages growth phases and burst size were determined as described previously[33]. A known number of an exponential-phase culture (ca. 1×10^7 CFU/ml) of each bacterial host was infected with each specific phage individually at an MOI of 1, phages were allowed to adsorb for 5 min at room

temperature. The mixture was then centrifuged at 5,000 rpm for 5 min and the supernatant was decanted to remove free-unbound phages. The bacteria-phage pellet was then washed twice and resuspended in 10 ml of TSB and maintained at 37°C with continuous shaking. At appropriate times phage titers were enumerated using plaque assay[35].

Determination of the bacteriophages host range

The host range for the two isolated phages vB_SauM-EG-AE3 and vB_EfaP-EF01 was determined against a collection of twenty-two bacterial isolates (Table S2, Supplementary data) as previously described with some modifications[36,37]. Ten microliters of each phage suspension (about 10^8 PFU/ml) were spotted, in duplicate, onto the TSA bacterial lawn plates and incubated at 37 °C for 16–18 h.

Thermal and pH stability

Thermal and pH-stability of the isolated phages were tested as described before[33]. For the assessment of thermal stability, 900 µl of pre-heated 0.22 µ m filter-sterilized SM buffer (5.8 g NaCl, 2.0 g $MgSO_4 \bullet 7H_2O$, 50 ml 1 M Tris-HCl pH 7.4, in 1-liter dH_2O) were added to 100 µl of each of phage lysates (8 log10 PFU/ml). Tubes were incubated at 10°C, 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C for 1 h. Aliquots were collected After 60 min of incubation to determine phage titers. For pH-stability assessment, phage lysates (8 $\log_{10}$ PFU/ml) were added to tubes containing sterile SM buffer with pH values ranging from 2-13 adjusted with NaOH and HCl. The tubes were incubated at 37°C for 60 min. Subsequently, the phage solutions were serially diluted and the recovered phage titers were determined using bacterial hosts employing the double-layer agar method[35]. Each temperature and pH treatment was performed in triplicates and the average of triplicate counts was calculated.

Phage thermal/pH stability (%) = (Recovered phage titers following the treatment / Initial Phage titer before treatment) ×100%

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Viral protein profiles were assed to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using Agilent 2100 Bioanalyzer system (Agilent Technologies, Cat # G2939BA) which is a chip-based automated electrophoretic assay. Solutions and reagents were prepared according to the Agilent High Sensitivity Protein 230 Kit manual (Reference number 5067-1517). The protein assay was performed following the manufacturer's instruction. The ladder ranged from 6 kDa (lower marker) to 240 kDa (upper marker). Results were analyzed and visualized using the 2100 Expert software provided by Agilent Technologies, USA.

Extraction of bacteriophages DNA and restriction analysis

DNA was extracted from purified high-titer stocks of phage using Wizard® Genomic DNA Purification Kit (Promega Corporation, Cat #A1120) according to the manufacturer's instructions. DNA integrity was checked using 1% agarose gel electrophoresis and the image was captured using a gel documentation system (Gel Doc. BioRad). Concentration and purity of purified DNA were measured by BioTek Epoch2 Microplate reader (Thermo Scientific, USA). For all samples, DNA purity was >1.8 ± 0.20 under absorbance ratio A260/A280.

For profiling and comparing bacteriophage genomic DNA fragment patterns, genomic DNA was digested with different restriction enzymes (BamHI, EcoRI, DraI, PstI, and KpnI). The digestion process was performed following the manufacturer's instructions of Promega Corporation for each enzyme. Restriction fragments were separated by electrophoresis using Agilent 2100 Bioanalyzer assay (Agilent Technologies, Cat # G2939BA) and following the manufacturer's instructions of Agilent High Sensitivity DNA kit (Cat # G2938-90321). The ladder sizing range varied from 50 bp to 10380 bp for size determination of DNA fragments. Results were analyzed and visualized using the 2100 Expert software provided by Agilent Technologies, USA.

Biocontrol of dental caries-causing bacteria using bacteriophages

The efficacy of phages to inhibit the growth of their hosts was assessed in broth medium using different MOIs. Phages were separately mixed with bacterial suspension that was diluted to10^3 CFU/ml from an overnight culture of *S. aureus* and *E. faecalis* to obtain MOIs of 10^3, 10^2, and 10 incubated at 37°C for 24 h. Times of collecting samples were at 1, 3, 5, and 24 h. Surviving bacterial cells were counted using

serial dilutions in sterile saline. Then, dilutions were plated onto nutrient plates and incubated at 37°C for 24 h. Bacterial growth was measured by monitoring optical densities at 600 nm.

RESULTS

Bacteria and antibiotic sensitivity testing

In the current study, *S. aureus* and *E. faecalis* were isolated formerly from infected patients with dental decays and cavities. The isolates were identified biochemically using conventional methods (Table S1, Supplementary data) and were confirmed by Vitek 2 system.

An antibiotic sensitivity test was performed for the two isolated bacteria against a selection of twelve antibiotics (Fig. S1, Supplementary data). Qualitative data from the antibiograms (Table 1) revealed that both*S. aureus* EG-AE3 and *E. faecalis* EF01 were resistant to at least eight antibiotics with a resistance percentage of 75% and 66%

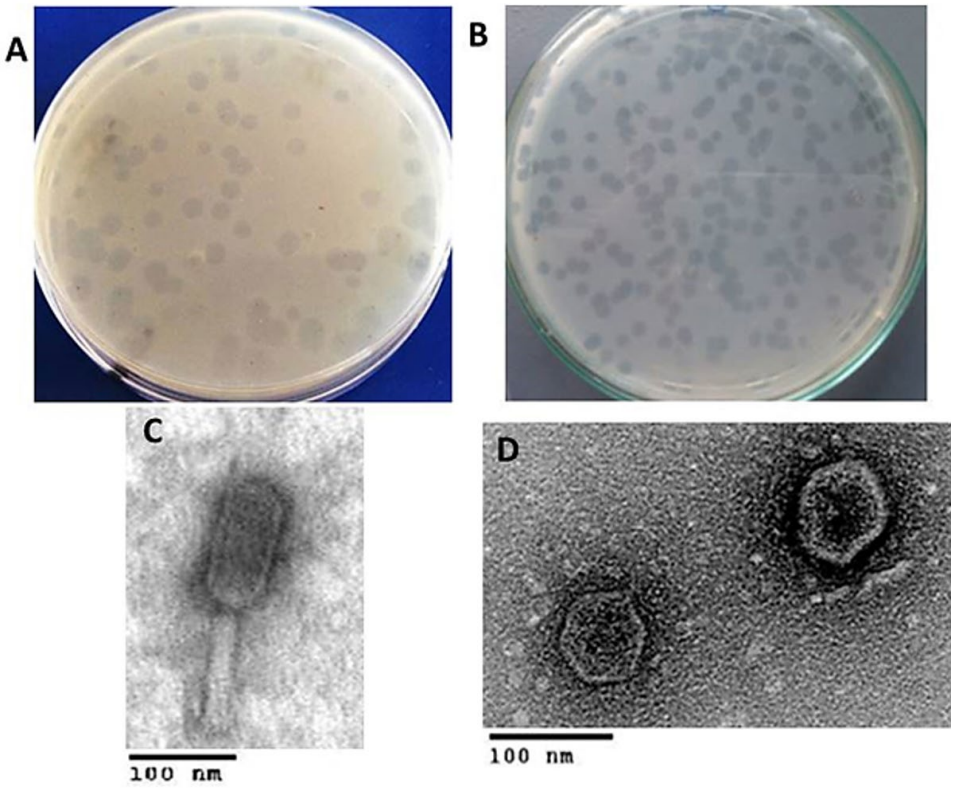

Fig. 1. Plaques phenotypes and TEM morphology of the isolated phages. (A-B) Images of bacterial plaques formed by the isolated phage in top-agar lawns of S. aureus EG-AE3 and E. faecalis EF01, plaque appearance was detected and imaged after culturing 48 h on their hosts. (C-D) TEM micrographs of phages vB_SauM-EG-AE3 and vB_EfaP-Ef01 were negatively stained with 0.2% uranyl acetate as described in Materials and Methods. Scale bar= 100 nm.

Table 1. Antibiotic sensitivity pattern of the isolated dental caries bacteria against a selection of twelve antibiotics

Bacteria	F	AX	C	CIP	GN	NOR	P	VA	CAZ	ATM	CTX	AK
S. aureus EG-AE3	R*	R	S*	I*	R	R	R	R	R	S	R	R
E. faecalis EF01	S	S	R	S	R	R	R	S	R	R	R	R

Nitrofurantoin (F, 10 µg), Amoxicillin (AX, 25 µg), Chloramphenicol (C 30 µg), Ciprofloxacin (CIP 5 µg), Gentamicin (GN, 10 µg), Norfloxacin (NOR, 10 µg), Penicillin-G (P, 10 µg), Vancomycin (V, 30 µg), Ceftazidime (CAZ, 30 µg), Aztreonam (ATM, 30 µg), Cefotaxime (CTX, 30 µg) and Amikacin (AK, 30 µg). * Denotes for Resistant (R), Intermediate (I), and Susceptible (S).

against the tested antibiotics, respectively. *S. aureus* resisted Nitrofurantoin, Amox0icillin, Gentamicin, Norfloxacin, Penicillin-G, Vancomycin, Ceftazidime, Cefotaxime, and Amikacin but was sensitive to Chloramphenicol and Aztreonam. *E. faecalis* EF01 resisted Chloramphenicol, Gentamicin, Norfloxacin, Penicillin-G, Ceftazidime, Aztreonam, Cefotaxime, and Amikacin. While, this isolate was susceptible to Nitrofurantoin, Amoxicillin, Ciprofloxacin, and Vancomycin.

Morphology of bacteriophages

Two phages with different plaque morphologies (Fig. 1A & B) targeted dental caries causing bacteria, *S. aureus*, and *E. faecalis* were successfully obtained after a screening of 100 samples of saliva. Successive double soft-layer agar assays led to pure phages isolation,titrated and processed at 4°C. Fig. 1 shows clear plaques produced by vB_SauM-EG-AE3 and vB_EfaP-EF01 on *S. aureus* EG-AE3 and *E. faecalis* EF01 as host strains respectively. Phage vB_SauM-EG-AE3 produces large circular clear plaques with a diameter of 4 mm and large circular clear plaques were formed by vB_EfaP-EF01 but with 3 mm in diameter. The concentrations of phages were $2.5x10^{10}$ PFU/ml to $3.1x10^{9}$ PFU/ml for vB_EfaP-EF01 and vB_SauM-EG-AE3 respectively.

Transmission Electron microscopy (Fig. 1C & D) allowed us to infer that vB_SauM-EG-AE3 and vB_EfaP-EF01 belong taxonomically to order Caudovirales. Dimensions of the isolated phages were measured and summarized in Table 2. The particle of vB_SauM-EG-AE3 had a contractile tail with 75 ±2 nm in length and head with a diameter of about 67 ±1 nm a typical member of Myoviridae family. While vB_EfaP-EF01 was a member of Podoviridae family whereas, the tail length was about 9 ± 0.5 nm and head diameter of about 58 ± 2 nm.

Growth-kinetics and host range

One-step growth kinetics of the isolated phages (Fig. 2) exhibited typical growth kinetics of most bacteriophages. Phages vB_SauM-EG-AE3 and vB_EfaP-EF01 gave burst sizes of 78.87 and 113.55 PFU/cell respectively with latent periods of 25 and 30 minutes. The host range of the isolated phages was estimated, and results were summarized in Table 3. Both phages established a narrow spectrum of lytic activity.

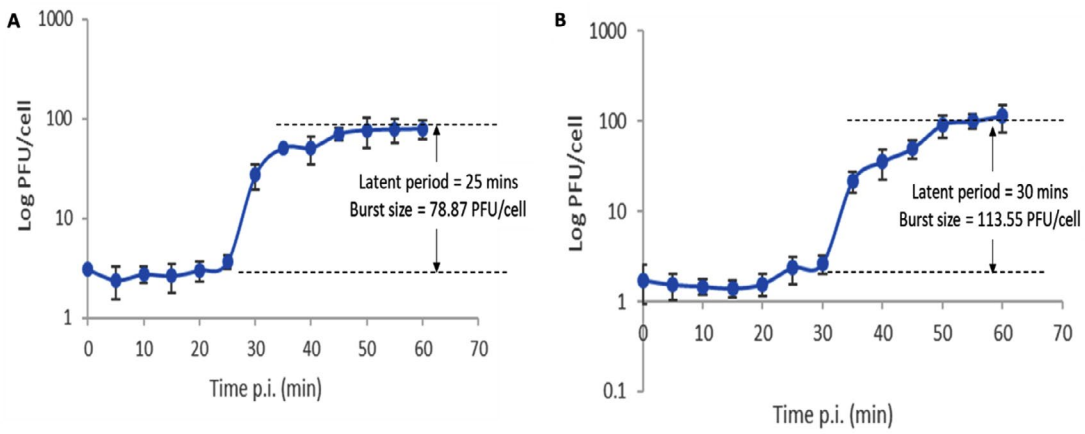

Fig. 2. One-step growth curves of phages vB_SauM-EG-AE3 (A) and vB_EfaP-Ef01 (B) on their corresponding hosts. Data shown are the mean of three replicates and error bars show the deviation in the values.

Table 2. Dimensions of the isolated phages

Phage	Bacterial host	Plaques diameter (mm)	Head diameter (nm)	Tail length (nm)	Proposed family
vB_SauM-EG-AE3	*S. aureus* EG-AE3	4	67 ± 1	75 ± 2	*Myoviridae*
vB_EfaP-Ef01	*E. faecalis* EF01	3	58 ± 2	9 ± 0.5	*Podoviridae*

Thermal and pH stability

Thermal and pH stability patterns of phages vB_SauM-EG-AE3 and vB_EfaP-EF01 were tested based on residual phage titers after incubation under different pH values and temperatures (Fig. 3). The *S. aureus* phage (vB_SauM-EG-AE3) was thermostable at temperatures ranging from 10 to 60 °C, but no viable phages were detected after heating at ≥70°C for 60 min. Phage vB_SauM-EG-AE3 resisted a pH range between pH 4 and 11 for 1h. Similarly, no significant reduction in the *E. faecalis* phage (vB_EfaP-EF01) was observed at temperatures ranging from 10 to 60°C. But, after heating at 70°C for 60 min, phage titers decreased by 60% and no viable phages were detected after heating at 80°C for 60 min.

Regarding the pH stability, both vB_SauM-EG-AE3 and vB_EfaP-EF01 phages were found to be resistant at a pH range of 4-11 after 60 min at 37 °C h (Fig. 3C-D).

Structural protein profiles

Molecular weights of bacteriophages' structural polypeptides ranged from 10 to 81 kDa (Fig. 4A). Phages vB_SauM-EGAE3 and vB_EfaP-Ef01 harbored major structural proteins of 39 and 44 kDa, respectively. Additionally, four minor structural proteins were observed in each phage where three of them revealed similar profiles in vB_SauM-EGAE3 and vB_EfaP-Ef01, each phage-displayed unique specific band at varied position of 81 kDa and 32 kDa for vB_SauM-EGAE3 and vB_EfaP-Ef01, respectively.

Restriction digestion patterns

Phages vB_SauM-EGAE3 and vB_EfaP-EGAE1 exhibited different patterns of restriction digest profiles (Fig. 4B). The restriction endonucleases of BamHI, EcoRI, DraI, and PstI were able to digest the two genomes. While KpnI failed to cut both genomes (Fig. S2, Supplementary data), the digestion of bacteriophage genomes with DraI showed the most crowded pattern with total fragments of 15 bands for vB_EfaP-EGAE1 and 9 bands for vB_SauM-EGAE3. On other hand, BamHI showed the lowest digestion profile with 3 bands for vB_SauM-EGAE3 and 4 bands for vB_EfaP-EGAE1. The restriction enzyme patterns

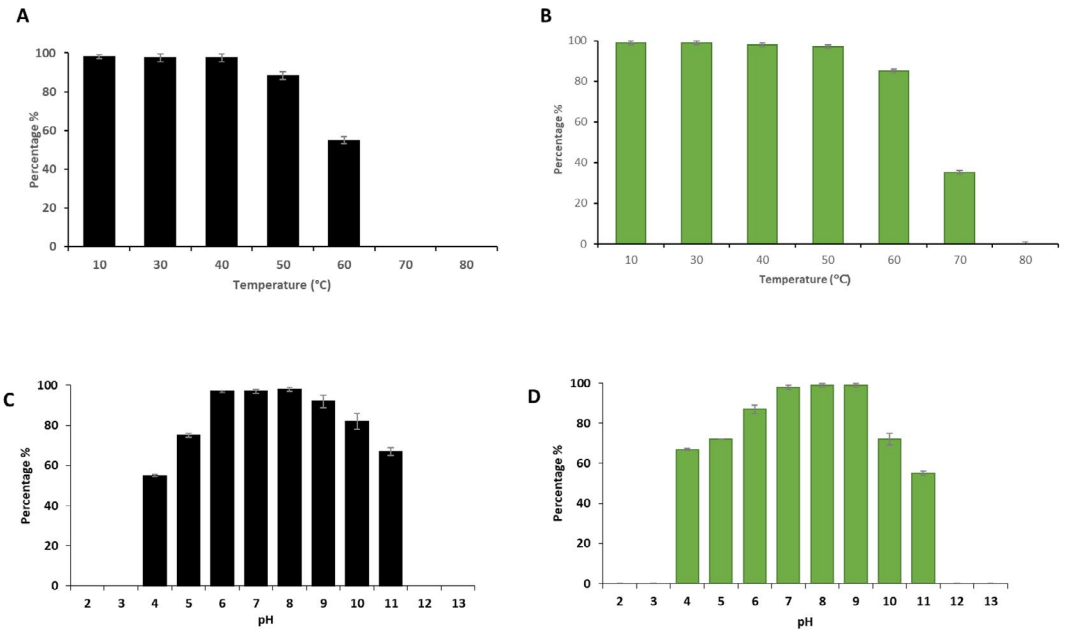

Fig. 3. Thermal and pH tolerance test of phages vB_SauM-EG-AE3 and vB_EfaP-Ef01. (A-B) Thermal tolerance, and (C-D) pH stability of vB_SauM-EG-AE3 and vB_EfaP-Ef01 phages, respectively. Temperature experiments were performed for 1 h at pH 7. pH tolerance was performed for 60 min at 37°C. Data showed the percentages of the remaining phages after each treatment as normalized from the control. Data shown are the mean of three replicates and error bars show the deviation in the values.

confirmed that these phages are double-stranded DNA viruses. For each phage, the genome size was predicted considering the total of the sizes of all fragments produced by restriction enzymes(Table S3, Supplementary data). The genome sizes were approx. 13.30 Kb and 15.60 Kb for phages vB_SauM-EGAE3, vB_EfaP-Ef01 respectively.

Biocontrol of dental caries-causing bacteria using the bacteriophages

In the current study, different MOIs were used to control targeted pathogens in broth medium (Fig. 5). High values of MOI were used to be more enough to infect bacterial cells and to reduce the chance of bacterial cells regrowth. In broth medium, complete inhibition of bacterial growth was achieved using phages with higher and lower MOIs of 10^3, 10^2 and 10 after 1, 3, 5, and 24 hr at 37°C compared to control counts. Based on the stability and lytic activity of the isolated phages against *S. aureus* and *E. faecalis*, they were applied to control such hosts in broth medium using different MOIs. Findings showed that MOIs of 10^3, 10^2 and 10 appeared efficient to eradicate the bacterial growth and prevent their regrowth in broth medium. Thus, the involvement of these phages in therapy could be promising as alternatives to antibiotics.

DISCUSSION

Dental caries is one of a hygiene-related disease caused by decay-causing bacteria that produce acid resulting in damage in tooth enamel[38]. In the current study, two pathogenic bacteria, *S. aureus* and *E. faecalis*, which are related to dental caries, were isolated previously from infected patients with dental decays and cavities, which agrees with Ohara-Nemoto et al.[39] and Wang et al.[40]. The isolated bacterial candidates were characterized microscopically and identified biochemically using conventional methods and were confirmed by Vitek 2 system according to previous studies[41,42].

Recently, most bacteria have the potential to develop resistance against different classes of antibiotics. Antibiotic resistance is one of the top concerns that threaten global health[43]. Egypt is one of the countries where antibiotic remedies have fewer extreme limitations[44,45] that enhances the chance for bacteria to resist antibiotics. In the current study, antibiotic sensitivity testing of *S.*

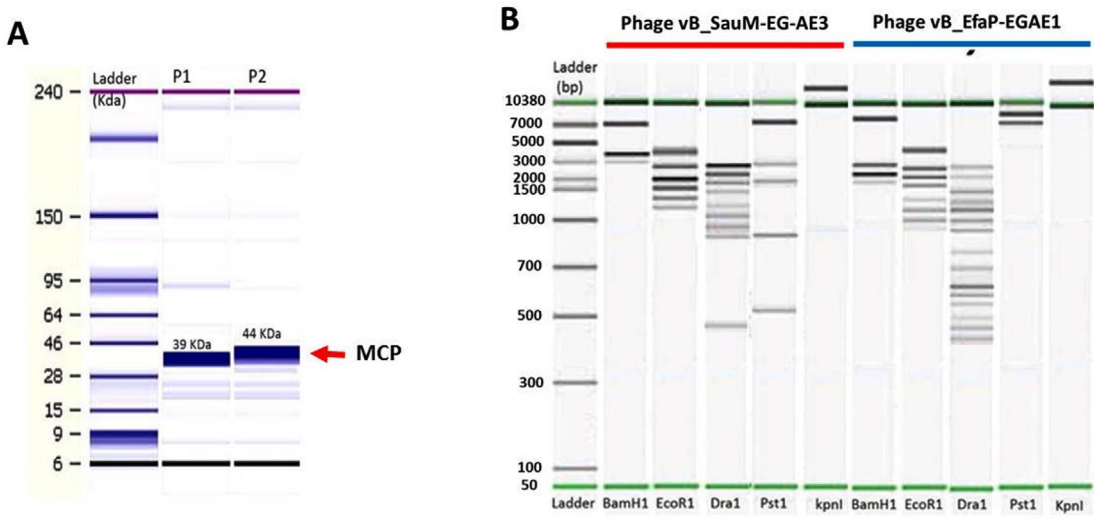

Fig. 4. Agilent bioanalyzer gel-like images of (A) SDS-polyacrylamide gel electrophoretic profiles of vB_SauM-EG-AE3 and vB_EfaP-Ef01 structural proteins. Lane1: ladder ranged from 6 kDa to 240 kDa, lanes 2-3: Structural protein profiles of vB_SauM-EG-AE3 and vB_EfaP-Ef01 respectively, red rows represent the major capsid proteins of the isolated phages. (B) Restriction digestion profiles of the vB_SauM-EG-AE3 and vB_EfaP-Ef01 phages after digestion of DNA with BamHI, EcoRI, DraI, PstI, and KpnI. Lanes as shown on the figure, Ladder from 50 bp to 10380 bp.

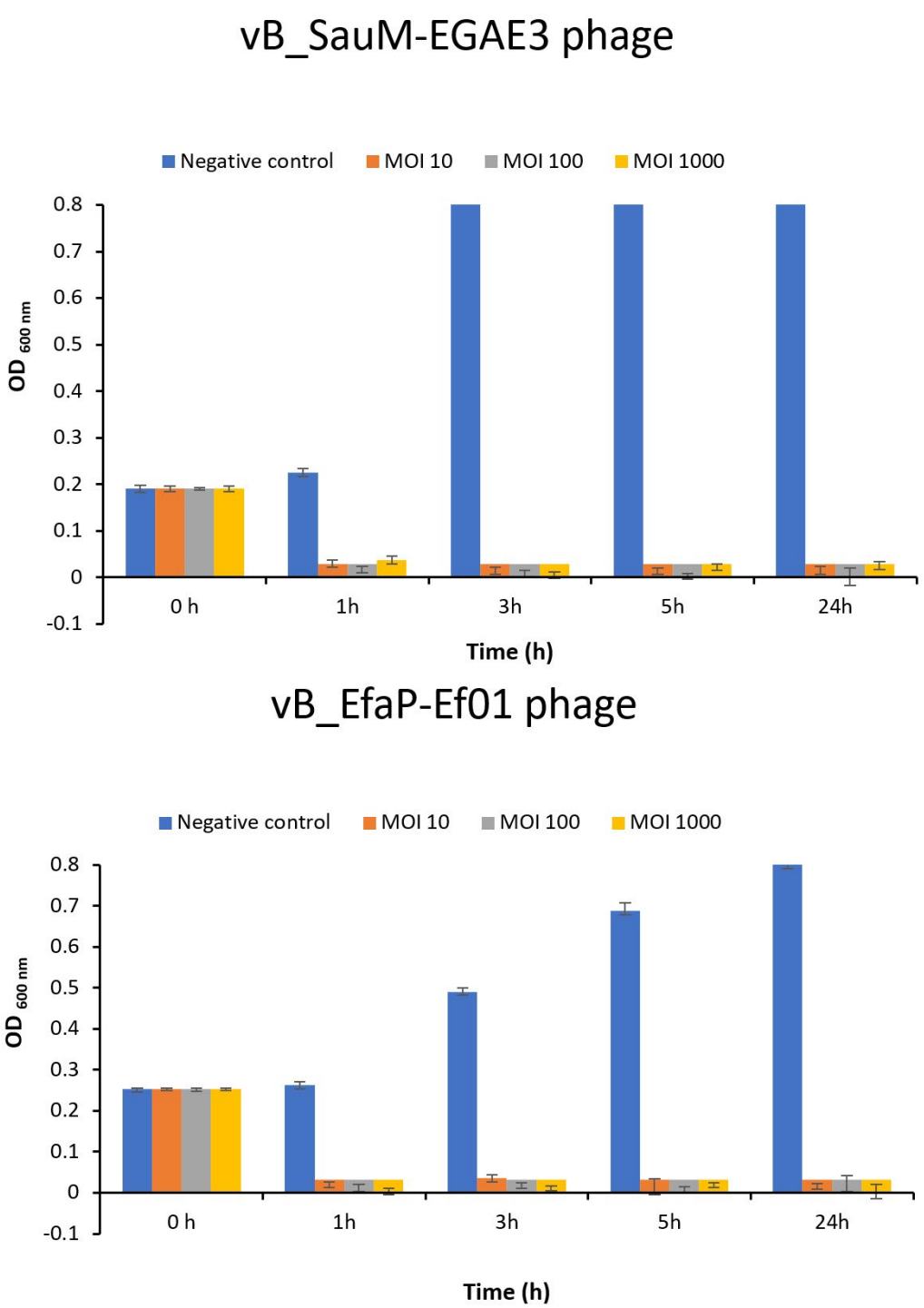

Fig. 5. *In vitro* biocontrol assay of the isolated phages on their corresponding hosts at different MOIs. (A) Lytic activity of vB_SauM-EGAE3 phage on *S. aureus* EG-AE3, and (B) Lytic activity vB_EfaP-Ef01 phage on *E. faecalis* EF01. Each bacteria was challenged with the corresponding phage at different MOIs of 10, 102, and 103. Bacterial growth was determined by measuring the optical density at 600 nm.

aureus and *E. faecalis* against a selection of twelve antibiotics showed that the two isolates resisted at least eight of the tested antibiotics. Resistance mechanisms against antibiotics by *S. aureus* and *E. faecalis* were reported[46,47].

Antibiotic resistance can be developed through mutations in chromosomal genes or by mobile genetic elements (horizontally acquired resistance)[48]. In that view, a resistance that is acquired through mutation, mechanism of horizontally acquired resistance, or overexpression of the drug efflux were discussed previously[47]. Hence, the current study used bacteriophages as an alternative strategy to control the spread of these organisms and mitigate dental caries.

Bacteriophages have been sought as one of the novel therapeutic approaches to control antibiotic-resistant pathogenic bacteria[49,50]. In the current study, two different phages (vB_SauM-EG-AE3 and vB_EfaP-EF01) targeted dental caries causing bacteria, *S. aureus*, and *E. faecalis*, respectively were isolated after a screening of 100 samples of saliva in concordance with previous study[51]. Phage vB_SauM-EG-AE3 belongs to the family Myoviridae, while vB_EfaP-EF01 belongs to Podoviridae.

Growth kinetics of the isolated phages exhibited typical growth kinetics of most bacteriophages. Phages vB_SauM-EG-AE3 and vB_EfaP-EF01 gave burst sizes of 78.87 and 113.55 PFU/cell respectively with latent periods of 25 and 30 minutes. These values, similar to those obtained in other studies with Staphylococcus and *E. faecalis* phages[52,46,40] where the average burst size of such phages was approximately 36-122 PFU/ infected cell and latent period 25-50 min. additionally, a recent study showed a higher burst size of *E. faecalis* phage of 352 PFU/ infected cell[21]. Both vB_SauM-EG-AE3 and vB_EfaP-EF01 phages displayed a narrow spectrum of lytic activity. This could be attributed to the ability of the tested strains to develop resistance against these phages[53,54]. Narrow host range could be overcome using a cocktail of phages[55]. In addition

Table 3. Host ranges of the isolated phages

Species	Strain ID number	Lysis by bacteriophage	
		vB_SauM-EG-AE3	vB_EfaP-Ef01
S. aureus	EG-AE3	+	-
	EG-AE1	+	-
	EG-AE2	-	-
	SA101	-	-
	SA1E	-	-
	BUH	-	-
E. faecalis	Ef01	-	-
	Etfc1	-	+
	Etfc2	-	+
	Etfc3	-	-
Enterococcus faecium	1	-	-
	2	-	-
	3	-	-
Acinetobacter baumannii	Acint1	-	-
	Acint2	-	-
	Acint3	-	-
E. coli	BE1	-	-
	BE2	-	-
	BE3	-	-
Proteus spp.	Pr1	-	-
Salmonella spp.	Sa1	-	-
Shigella spp.	Sh1	-	-

"–," no clearing; "+," completely clear.

to that cross infectivity of phages against different species and genera was investigated in the current study, and no lytic activity was shown.

The stability of phages under stressful environmental conditions promotes the application of phages as a bio-control agent in dental therapy. At temperatures between 10 and 60°C,and pH range between pH 4 and 11 for 1h.*S. aureus* phage was stable. Interestingly, no significant reduction in the *E. faecalis* phage was observed at temperatures ranging from 10 to 70° C or pH from 7 to 9. Previous studies showed stability of phages against *S. aureus* and *E. faecalis* under a wide range of temperature degrees (4–60°C) and resistancein acidic or alkaline pH (pH 3–12)[21,56]. These findings make the isolated phages highly potent to be used in clinical settings where they can be combined with alkaline disinfectants that are commonly used in the treatment of endodontic infections.

In the protein fractions obtained in the current study for two phages we detected the occurrence of main fractions with a molecular weight ranged from 10 to 81 kDa and the number of bands obtained ranged from 4 and 5 fractions for vB_SauM-EGAE3 and vB_EfaP-EGAE1 respectively. Seven protein bands were visualized on SDS-PAGE for purified *S. aureus* phage vB_SauS_SA2. The protein band of about 33 kDa was identified to be the major capsid protein by mass spectrometry, which is relatively smaller than the capsid protein (42 kDa) of phage SA2[57]. While, SDS-PAGE analysis of the dissociated purified *E. faecalis* phage φEf11showed 11 well-resolved bands of proteins ranging from 27 to 85 kDa in size[58]. In taxonomic and phylogenic classifications, major capsid proteins, highly conserved among viruses, are often used.[59,60]. So, the close relation of vB_SauM-EGAE3 and vB_EfaP-Ef01 to the other phages of Gram-positive bacteria can, therefore, be speculated on.

Phages vB_SauM-EGAE3 and vB_EfaP-EGAE1 showed different patterns of restriction digest profiles and the genome sizes were approx. 13.30 Kb and 15.60 Kb for phages vB_SauM-EGAE3, vB_EfaP-Ef01 respectively. Kwiatek et al.[61] characterized a virulent *S. aureus* phage (MSA6) which isolated from cow milk with mastitis and they indicated that the genome size of MSA6 of about 143 Kbp based on the KpnI and PstI restriction pattern and PFGE. Uchiyama et al.[62] reported that the genome size of *E. faecalis* phage φEF24C was estimated to be about 143 kb. Phages vB_SauM-EGAE3 and vB_EfaP-EGAE1 were refractory for endonuclease enzyme digestion, such as KpnI. Interestingly, this mechanism, called an anti-restriction mechanism, is usual among phages of the *Siphoviridae*[63,64] and *Myoviridae*[65].

Phages developed various strategies of anti-restriction against restriction-modification (R-M) systems. Protection against DNA bacteriophages is given by staphylococcus (R-M) systems. These systems consist of methyltransferase DNA and endonuclease restrictions, which through methylation, protect self-DNA and inhibit foreign unmodified DNA in the same sequence. If the oligonucleotide sequence recognized by the enzyme is found, a DNA molecule will be hydrolyzed by the restriction endonucleases. As a consequence of point mutations or the acquisition in their genomes of the cognate methylase gene, a lack of endonuclease recognition sites in their genomes may be the strategy used by phages. Generally, methylation within the recognition sequence of one specific cytosine (C) or adenine (A) residue will prevent sequence cleavage by Type II restrictionendonuclease[66,67].

Based on the stability and lytic activity of the isolated phages against *S. aureus* and *E. faecalis*, they were applied to control such hosts in broth medium using different MOI. Findings showed that MOI 10, 100 and 1000 appeared efficient to eradicate the bacterial growth and prevent their regrowth in broth medium. Thus, the involvement of these phages in therapy could be promising as alternatives to antibiotic. Using bacteriophages for the prophylaxis or treatment of oral infectious diseases was adopted in previous studies[68,69,50]. ListShieldTM and LISTEXTM P100, commercial phages, are approved to use and have gotten GRAS, Generally Recognized As Safe, status through FDA[70], also a recent review studied possibilities of using bacteriophages against pathogenic bacteria with oral origin and found possible future uses of phages in dentistry[71]. A few promising studies attempted to use phages for treatment of dental infections caused by bacterial pathogens like *S. aureus*[72] and *E. faecalis*[28]. Phages are overseen as a friendly biocontrol strategy and can be granted a commercial use. For example,

ListShieldTM and LISTEXTM P100, commercial phages, are approved to use and have gotten GRAS status through FDA[70]. As a future work, we are trying to isolate more bacteriophages against dental caries causing bacteria and to produce a phage cocktail mix to be incorporated. Later this phage mix suspension can be used while dental flossing to reduce or eliminate dental caries bacteria into the interdental spaces. Moreover, the phage cocktail could be loaded on small sweety pleasant tablets that can be used daily, or merged with chewing gums and/ or incorporated on toothpaste.

CONCLUSION

Health concerns related to dental diseases have been exacerbated by antibiotic-resistant *S. aureus* and *E. faecalis*. Hence, bacteriophages could be proposed as an alternative strategy to mitigate the causative bacterial pathogens. In this study, two virulent phages were isolated for antibiotic-resistant *S. aureus* and *E. faecalis*, vB_SauM-EG-AE3belonged to family *Myoviridae* and vB_EfaP-EF01belonged to family *Podoviridae* morphologically. The two phages have narrow host ranges, low latent periods, strong pH and thermal resistance. More importantly, our findings demonstrate the efficacy of phages vB_SauM-EG-AE3 and vB_EfaP-EF01 for the inhibition of multidrug-resistant *S. aureus* and *E. faecalis* growth *in vitro* respectively. This research forms the basis for the therapeutic application of phages to manage*S. aureus* and *E. faecalis* infection.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.15.1.XX

Additional file: Additional Table S1- S3. Additional Figure S1-S2.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AE designed and directed the project. MAN, NKE, YHE and AE performed the Experiments. MAN, NKE and AE analyzed the data. MAN, NKE and AE wrote the Initial draft. AE reviewed and edited the final manuscript. All authors discussed the Results and commented on the manuscript.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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